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Interaction of serologically defined colon cancer antigen-3 with Arf6 and its predominant expression in the mouse testis

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ABSTRACT

ADP ribosylation factor 6 (Arf6) is a small GTPase that regulates endosomal trafficking and actin cytoskeleton remodeling. Here, we identified the serologically defined colon antigen-3 (SDCCAG3) as an Arf6-interacting protein by yeast two-hybrid screening with a constitutively active Arf6 mutant. SDCCAG3 interacts specifically with Arf6 among the Arf family members through its 101 C-terminal amino acids. SDCCAG3 is expressed most intensely in the testis at the mRNA and protein levels. In the testis, SDCCAG3 is expressed in spermatocytes and spermatids. We also show that full-length SDCCAG3, but not a mutant lacking the ability to interact with Arf6, is recruited to the midbody during cytokinesis when expressed exogenously in HeLa cells. These findings suggest that SDCCAG3 might function in endosomal trafficking downstream of Arf6.

1. Introduction

ADP ribosylation factor 6 (Arf6) belongs to the Arf small GTPase family and localizes at the plasma membrane and endosomes where it regulates actin cytoskeleton remodeling and membrane trafficking by cycling between GDP-bound inactive and GTP-bound active states. Arf6 is implicated in a variety of cellular events related to morphological changes such as cytokinesis, migration, phagocytosis, endocytosis, exocytosis, and neurite extension [1–3]. Among these, cytokinesis is the division of the cytoplasm of a single cell to form two daughter cells at the final step of mitotic cell division, and represents an extreme example of cell phenomena accompanying drastic changes in cell shape. In addition to involvement of the actin cytoskeleton, membrane trafficking is known to play an essential role in cytokinesis through the addition of new membrane to compensate for the massive increase in surface area, and the local enrichment of protein and lipid components required for cleavage furrow ingression and abscission. Since an initial report showing the localization of a constitutively active Arf6 mutant at the cleavage furrow and midbody and the activation of Arf6 during cytokinesis [4], accumulating evidence has established Arf6 as an important regulator of cytokinesis. Several effectors of Arf6 during cytokinesis have been so far identified including Rab11 family-interacting proteins FIP3/4 [5], Sec10 subunit of the exocyst complex [5,6], c-Jun N-terminal kinase-interacting proteins JIP3/4 [7], and mitotic kinesin-like protein MKLP1 [8,9]. Through its interaction with these effectors, Arf6 is proposed to regulate various steps of cytokinesis, including endosomal transport along central spindle microtubules [7], tethering and fusion of transport vesicles carrying components required for cleavage furrow ingression and abscission [5,10], and maintenance of structural integrity of the intercellular bridge and midbody [8,9].

The serologically defined colon cancer antigen-3 (SDCCAG3) is an endosomal protein that was originally isolated by screening of cDNA expression libraries from human colon cancers with autologous patient antibodies [11]. Inhibition of SDCCAG3 activity by overexpression of the C-terminal region containing a coiled-coil domain was shown to decrease the presentation of tumor necrosis factor (TNF) receptor-1 on the cell surface and to protect cells from TNF-induced apoptosis, suggesting the involvement of SDCCAG3 in membrane trafficking [12]. Indeed, SDCCAG3 localizes at a subpopulation of early/recycling endosomes that partially overlap with early endosome antigen 1 (EEA1) and the transferrin receptor [13]. Recent proteomic analysis revealed that SDCCAG3 forms a protein complex with the retromer complex through an interaction with the Wiskott–Aldrich syndrome protein and scar

Abbreviations: Arf, ADP ribosylation factor; EEA1, early endosome antigen 1; FIP, Rab11 family-interacting protein; GST, glutathione S-transferase; JIP, c-Jun N-terminal kinase-interacting protein; SDCCAG3, the serologically defined colon antigen-3; TNF, tumor necrosis factor; WASH, the Wiskott–Aldrich syndrome protein and scar homolog.

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homolog (WASH) complex [14] and regulates sorting nexin27-retromer-mediated endosomal trafficking of the glucose transporter GLUT1 [15]. During cytokinesis, SDCCAG3 undergoes dynamic subcellular changes and accumulates at the midbody [13]. SDCCAG3 was shown to regulate the completion of cytokinesis through its interaction with the protein tyrosine phosphatase PTPN13 and the Arf GTPase-activating protein GIT1 [13].

In this study, we identified SDCCAG3 as an Arf6-interacting protein by yeast two-hybrid screening with a GTP-locked Arf6 mutant as bait and characterized the tissue distribution of SDCCAG3 at the mRNA and protein levels. Furthermore, we provide evidence suggesting the importance of an interaction between SDCCAG3 and Arf6 in the midbody localization of SDCCAG3.

2. Materials and methods

2.1. Animals

We purchased male C57BL/6 mice at postnatal week 10 from CLEA Japan (Tokyo, Japan) for western blot, northern blot and immunohistochemical analyses, and female Hartley guinea pigs weighing 250–300 g from Japan Laboratory Animals, Inc. (Tokyo, Japan) for antibody production. All animal experiments were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine and conducted in compliance with the Animal Use Guidelines of the Kitasato University School of Medicine.

2.2. Plasmids

Arf mutants in two-hybrid bait vectors (pDBLeu) were generated as described [16]. The entire coding region and truncated cDNA fragments of mouse SDCCAG3 (accession number NM_001085407) were amplified by polymerase chain reaction (PCR) with a first-strand mouse brain cDNA library and specific primer pairs, in which sense and anti-sense primers were supplemented at their 5′ ends with a *Sal*I restriction site (underlined) and a stop codon (double-underlined), respectively, as follows:5′-GTCGACCATGTCGGGCTACGCGCGGCGGCAGG-3′ and 5′-TCAAGAGTCCACCTCATCTTTAAC-3′ for the entire coding region (full); 5′-GTCGACCATGTCGGGCTACGCGCGGCGGCAGG-3′ and 5′-TCAGGCTTCATTTTCTCGCCTCAAGTTTG-3′ for amino acids 1–316; 5′-GTCGACGATGACCAAACGGGCTGTAAAAGCAG-3′ and 5′-TCAGGCTTCATTTTCTCGCCTCAAGTTTG-3′ for amino acids 282–382; 5′-GTCGACGATGACCAAACGGGCTGTAAAAGCAG-3′ and 5′-TCAGGCTTCATTTTCTCGCCTCAAGTTTG-3′ for amino acids 282–316; 5′-GTCGACCCTGCGGTCAGGCCAGGGTGCCAGCC-3′ and 5′-TCAGGCTTCATTTTCTCGCCTCAAGTTTG-3′ for amino acids 317–382; 5′-GTCGACATCTGCCGTGGATCAGACTCATTCC-3′ and 5′-CTAGATCATCTTCGCCTCCAACTTCCG-3′ for amino acids 139–259. After PCR fragments had been subcloned into pGEM-Teasy (Promega, Madison, WI), the inserts were digested with *Sal*I and *Not*I restriction enzymes and subcloned into a two-hybrid prey vector pPC86 (Invitrogen, Carlsbad, CA), mammalian expression vector pCMV-Myc (Clontech Laboratories, Mountain View, CA), or the bacterial expression vectors pGEX4T-2 (GE Healthcare Bio-Sciences, Piscataway, NJ) and modified pMAL-2c (New England Biolabs, Beverly, MA) [17]. For pmCherry-N1-Arf6, the coding region of mouse Arf6 was amplified by PCR with primer pairs in which sense and anti-sense primers were supplemented at their 5′ ends with *Eco*RI (underlined) and *Sal*I (double-underlined) restriction sites (sense: 5′-GAATTCACCATGGGGAAGGTGCTATCCAAGATC-3′ and anti-sense: 5′-GTCGACTGGGATTTGTAGTTAGAGGTTAAC-3′). The PCR fragment was subcloned into *Eco*RI and *Sal*I sites of pmCherry-N1 (Clontech).

2.3. Yeast two-hybrid assay

Yeast two-hybrid screening of a mouse brain cDNA library with a constitutively GTP-bound Arf6 mutant, Arf6(Q67L), as bait has been described in detail previously [16,18]. Briefly, approximately 6×10^6 yeast colonies were screened by β-galactosidase activity, by prototrophy for histidine and uracil, and by 5-fluoroorotic acid (5-FOA) toxicity according to the manufacturer's protocol (ProQuest Two-Hybrid system; Invitrogen). Plasmids from positive colonies were isolated and subjected to sequencing.

2.4. Northern blot analysis

Total RNAs (20 μg) were isolated from various tissues of C57BL/6 mice at postnatal week 10 using Trizol (Invitrogen) and subjected to northern blot analysis with a ^{32}P-labeled cDNA fragment of the SDCCAG3 coding region as described [16].

2.5. Antibody production

The central region of SDCCAG3 corresponding to amino acids 139–259 was bacterially expressed as fusion proteins of glutathione S-transferase (GST) or maltose binding protein, and purified using glutathione-Sepharose 4B (GE Healthcare) or amylose-resin (New England Biolabs), respectively. The GST–SDCCAG3 fusion protein was conjugated with Freund's adjuvant and injected subcutaneously into guinea pigs five times at 2-week intervals. Sera were subjected to affinity purification using the maltose binding protein–SDCCAG3 fusion protein coupled to CNBr-activated sepharose 4B (GE Healthcare).

2.6. Western blot analysis

Total lysates (10 μg) were prepared by homogenizing various tissues and HeLa cells with a buffer consisting of 250 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol, and subjected to western blot analysis with anti-SDCCAG3 or anti-FLAG antibodies as described [18]. The membrane was reprobed with an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (CB1001, Merck Millipore, Darmstadt, Germany) as a loading control.

2.7. Immunostaining

HeLa cells were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. They were transfected with pmCherry-N1-Arf6, or pCMV-Myc vectors encoding full-length or truncated mutants of SDCCAG3 using Lipofectamine 2000 (Invitrogen) for 15–18 h and fixed in 4% paraformaldehyde prewarmed to 37 °C. Cells were solubilized with 0.3% Triton X-100 in phosphate-buffered saline, blocked with 5% normal donkey serum, and incubated overnight with guinea pig anti-SDCCAG3, rabbit anti-mCherry [19], mouse anti-α-tubulin (DM1A; Sigma-Aldrich, St. Louis, MO) antibodies for triple immunofluorescence staining or rabbit anti-c-Myc (C3956; Sigma-Aldrich) and mouse anti-α-tubulin antibodies for double immunofluorescence staining. Immunoreactions were visualized with an appropriate combination of species-specific secondary antibodies conjugated with Alexa Fluor 488, 594, or 647 (Invitrogen), and nuclei were counterstained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). For immunohistochemistry, three C57BL/6 mice were perfused transcardially with 4% paraformaldehyde. Tissues were postfixed in the same fixative for 1 h and immersed in 30% sucrose in phosphate-

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